RESEARCH COMMUNICATION

Kukoamine A and other hydrophobic acylpolyamines: potent and selective inhibitors of *Crithidia fasciculata* trypanothione reductase

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The enzyme trypanothione reductase (TR), together with its substrate, the glutathione–spermidine conjugate trypanothione, plays an essential role in protecting parasitic trypanosomatids against oxidative stress and is a target for drug design. Here we show that a naturally occurring spermine derivative, the anti-hypertensive agent kukoamine A [N^1N^{12}-bis(dihydrocaffeoyl)-spermine] inhibits TR as a mixed inhibitor ($K_i = 1.8$ μM, $K_{ii} = 13$ μM). Kukoamine shows no significant inhibition of human glutathione reductase ($K_i > 10$ mM) and thus provides a novel selective drug lead. The corresponding N^1N^8-bis(dihydro-caffeoyl)spermidine derivative was synthesized and acted as a purely competitive inhibitor with $K_i = 7.5$ μM. A series of mono- and di-acylated spermines and spermidines were synthesized to gain an insight into the effect of polyamine chain length, the nature and position of the acyl substituent and the importance of conformational mobility. These compounds inhibited TR with K_i values ranging from 11 to 607 μM.

INTRODUCTION

Many facultative intracellular parasites lack important oxygen-detoxifying enzymes such as superoxide dismutase and catalase which confer protection against oxidative stress [1,2]. For such pathogens, the glutathione-based antioxidant capabilities of the host cell are often quite important for survival [3,4]. However, trypanosomatid parasites such as *Leishmania*, *Trypanosoma cruzi*, *T. congolense*, *T. brucei* and *Crithidia fasciculata* are protected against oxidative damage by a glutathione-like system involving trypanothione (eqn. 1), a unique covalent conjugate of glutathione with spermidine [5,6]. By analogy with glutathione, the redox chemistry of trypanothione cycles between its oxidized, disulphide form [oxidized trypanothione, $T(S)_2$] and a reduced, dithiol, form [reduced trypanothione, $T(SH)_2$], which is the biologically active oxygen scavenger:

$$(1)$$
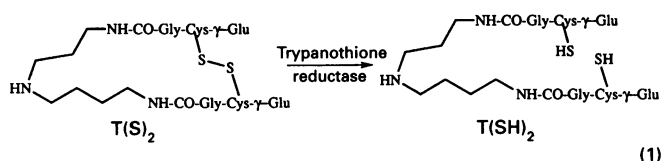
The regeneration of $T(SH)_2$ from $T(S)_2$ is catalysed by trypano-thione reductase (TR), an NADPH-dependent flavoenzyme [7,8] whose structure has been investigated by X-ray diffraction ([9–13]; C. L. Strickland and P. Karplus, unpublished work). Although amino-acid-sequence similarities and X-ray analysis reveal that both TR and human glutathione reductase (GR) [15,16] are similar (subunit $M_r \approx 50$ kDa), TR displays a 10^8-fold preference for trypanothione over glutathione, and human GR exhibits a 10^4-fold preference for glutathione [17]. Thus, selective inhibitors of TR represent an attractive target for the design of non-toxic, anti-parasitic drugs [17,18].

Human erythrocyte GR has been the subject of many enzymo-logical and crystallographic studies, and its catalytic apparatus is well understood [19]. Although oxidized glutathione (GSSG) is symmetric, it binds asymmetrically to GR with glutathione-I in GSSG (GS-I) more deeply situated in the active-site cleft. Both γ-glutamyl groups are specifically recognized, and the disulphide binds in the central portion of the site in a relatively hydrophobic pocket. The glycine carboxylate of GS-I interacts with an arginine, while that of glutathione-II in GSSG (GS-II) remains mobile and appears relatively unimportant for binding. In TR, the γ-glutamyl- and disulphide-recognition regions are highly similar to those of GR, but the region equivalent to that binding the glycine carboxylate is altered in charge and hydrophobicity to accommodate the polyamine bridge [17,19]. These differences likely explain the observed preference for cognate substrates.

Several promising new inhibitors of TR have recently been reported. Families of trypanocidal naphthoquinones [20] and nitrofurans [21] have been shown to subvert the action of TR by enzyme-catalysed redox cycling. The trypanocidal effect of organoarsenicals like melarsen oxide likely involves reaction with the dithiol $T(SH)_2$ to form an adduct which is a competitive inhibitor of TR [22,23]. A molecular-modelling study of known tricyclic anti-trypanosomal agents [24] identified three selective TR inhibitors, with the most active, clomipramine, having a K_i of 6 μM. Homology modelling of the TR active site also led to several peptide-based inhibitors, of which benzoyl-Leu-Arg-Arg β-naphthylamide was the most potent ($K_i = 13.8$ μM) [25]. Recently, several non-reducible analogues of $T(S)_2$ were synthesized, and these inhibited TR with K_i values between 30 and 90 μM [26]. Most of these inhibitors are molecules with a significant hydrophobic region and a net positive charge. This is consistent with knowledge of the binding site based on X-ray-diffraction studies, where the disulphide of trypanothione is bound in a central hydrophobic pocket and the spermidine moiety binds near a glutamic acid side chain.

Compound structure

Activity (K_i)

$K_i = 1.8 \, \mu$M
$K_{ii} = 13 \, \mu$M

1 Kukoamine A

7.5 μM

2

$K_i = 16 \, \mu$M
$K_{ii} = 68 \, \mu$M

3

43 μM

4

85 μM*

5

108 μM*

6

150 μM*

7

150 μM*

8

$K_i = 283 \, \mu$M
$K_{ii} = 1833 \, \mu$M

9
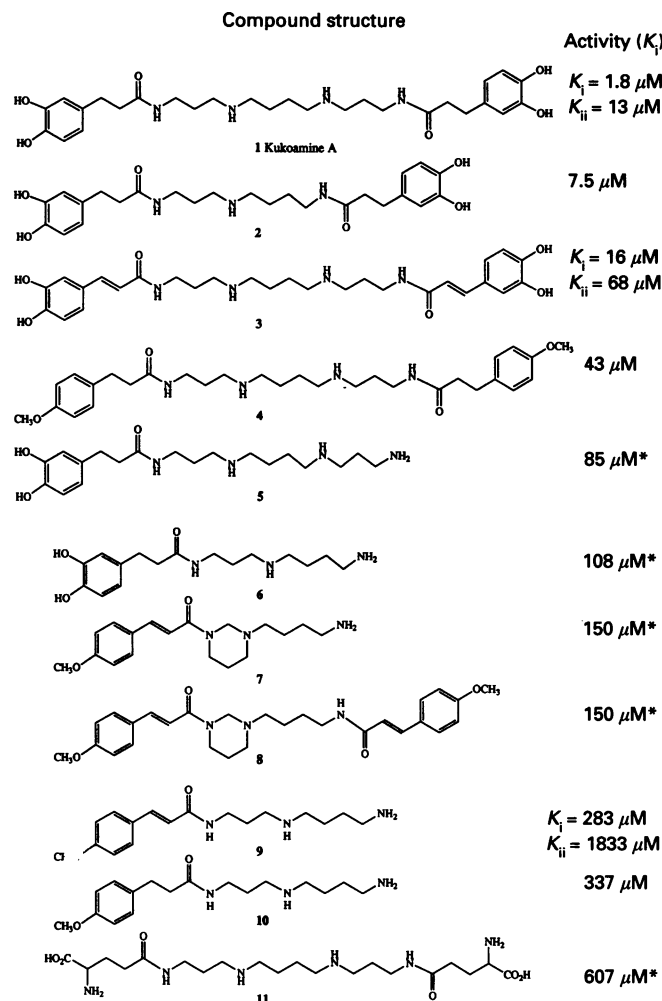
337 μM

10

607 μM*

11

Figure 1 Structures of kukoamine A and other acylated spermidines and spermines and their derivatives

The kinetic constants K_i and K_{ii} refer, respectively, to the dissociation constants of inhibitor from free enzyme and from enzyme–substrate complex. Asterisks indicate an estimated K_i. The kinetic parameters are defined in the Materials and methods section.

Our knowledge of the TR active site led us to consider the natural polyamine derivative kukoamine A (compound **1**, Figure 1), as a prospective inhibitor of TR. Kukoamine A, which is N^1N^{12}-bis(dihydrocaffeoyl)spermine, was first isolated from the root bark of *Lycium chinense*. Extracts of this plant have been used in oriental medicine to treat hypertension and stress ulcers. The active constituent, compound **1**, has been shown to be a clinically effective hypotensive agent [27], and two successful total syntheses have been reported [28,29]. Related diacylated polyamines, including N^1N^4-(biscaffeoyl)-1,4-diaminobutane, have been isolated from tobacco (*Nicotiana tabacum*) [30,31] and their presence established in other plants [32]. Here we report that kukoamine A is a potent inhibitor of TR and is devoid of significant inhibitory effects on GR. To evaluate structure–activity relationships, we further describe the synthesis and inhibitory properties of a series of acylated spermidine and spermine-based polyamines **1–11** (Figure 1), whose functionalities were designed to probe the optimal fit within the charged and hydrophobic binding pockets of the trypanothione-binding site in TR.

MATERIALS AND METHODS

Kukoamine A (compound **1**), was prepared according to a published total synthesis [33]. Syntheses of polyamines **2–11** are outlined in Scheme 1. The individual syntheses were completed using known methodology [28], and involved the specific sequences of intermediates shown in Scheme 2.

Representative examples of all the procedures employed in Scheme 1 are presented in the following detailed experimental description of the synthesis of inhibitor **6**. The structures of all intermediates were confirmed by IR, NMR and fast-atom-bombardment MS. Full details are available from the authors on request.

Syntheses

Synthesis of compound 13

To hexahydropyrimidine (compound **12**) (520 mg, 3.32 mmol) in distilled tetrahydrofuran (THF) (3.5 ml) at 0 °C was added BOC-ON [2-(t-butoxycarbonyloxyimino)-2-phenylacetonitrile (Aldrich); 782 mg, 3.2 mmol] in THF (13.3 ml) via a syringe over 30 min. After the solution had been stirred at 0 °C for 30 min, the solvent was removed *in vacuo*. The residue was dissolved in diethyl ether (50 ml) and washed with 2 × 10 ml of aq. 5% NaOH. The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated *in vacuo*. The residue was purified by flash chromatography (SiO_2; dichloromethane/NH_3-saturated methanol, 9:1, v/v) to yield compound **13** (364 mg, 43%) as an oil.
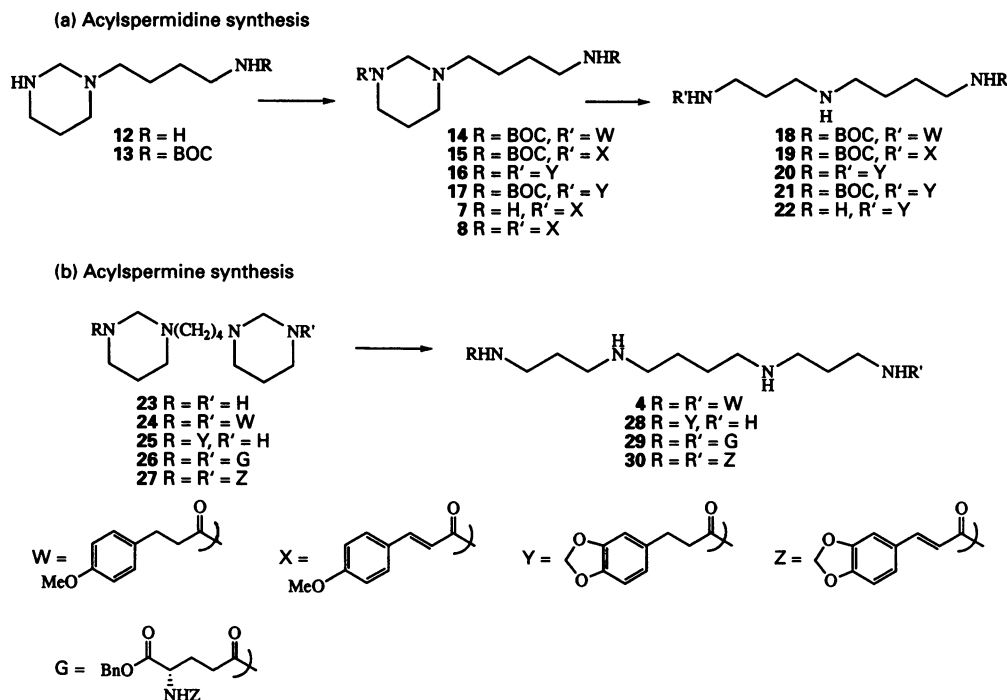
Synthesis of compound 17

To a stirred suspension of 3,4-(methylenedioxy)hydrocinnamic acid (195 mg, 1 mmol) in distilled dichloromethane (2.1 ml) was added 1,1′-carbonyldi-imidazole (162 mg, 1 mmol) in one portion. The suspension was stirred at room temperature under Ar for 1 h. A solution of compound **13** (257 mg, 1 mmol) dissolved in distilled dichloromethane (1 ml) was added, and the suspension stirred overnight. The reaction was diluted to 7.5 ml with dichloromethane and washed with saturated $NaHCO_3$ (7.5 ml). The aqueous layer was further extracted with 3 × 7.5 ml of dichloromethane. The combined organic layers were concentrated *in vacuo* to yield 359 mg of crude compound **17** as a yellow oil, which was carried on to the next step without purification.

Synthesis of compound 21

A suspension of compound **17** (433 mg, 1 mmol), ethyl hydrogen malonate (555 μl, 5 mmol) and pyridine (324 μl, 4 mmol) in 100% ethanol (25.7 ml) was stirred at room temperature for 30 min. The suspension was then heated at reflux for 2.5 h. The solvent was removed *in vacuo*. The residue was dissolved in 10 ml of saturated $NaHCO_3$, and extracted with 3 × 10 ml of dichloromethane. The combined organic layers were dried over anhydrous $NaSO_4$, filtered, and concentrated *in vacuo*. The resulting residue was purified by flash chromatography [SiO_2; dichloromethane/NH_3-saturated methanol (19:1, v/v) followed by dichloromethane/NH_3-saturated methanol (9:1, v/v)] to yield compound **21** (163 mg, 38%, two steps) as a tan-coloured solid (m.p. 82–84 °C).
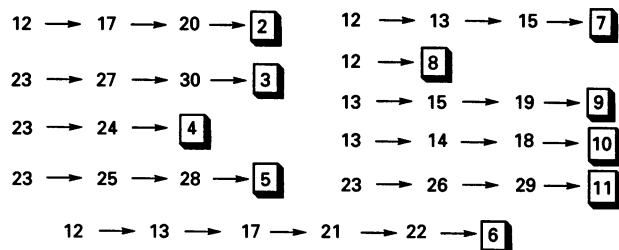
Synthesis of compound 22

To a solution of compound **21** (162 mg, 0.386 mmol) in distilled methanol (3.1 ml) was added 3 M HCl/methanol (1.28 ml,

(a) Acylspermidine synthesis

12 R = H
13 R = BOC

14 R = BOC, R' = W
15 R = BOC, R' = X
16 R = R' = Y
17 R = BOC, R' = Y
7 R = H, R' = X
8 R = R' = X

18 R = BOC, R' = W
19 R = BOC, R' = X
20 R = R' = Y
21 R = BOC, R' = Y
22 R = H, R' = Y

(b) Acylspermine synthesis

23 R = R' = H
24 R = R' = W
25 R = Y, R' = H
26 R = R' = G
27 R = R' = Z

4 R = R' = W
28 R = Y, R' = H
29 R = R' = G
30 R = R' = Z

W =

X =

Y =

Z =

G = BnO

Scheme 1   Overview of synthetic routes to acylspermidines (a) and acylspermines (b)

Abbreviation used: BOC, benzoyloxycarbonyl.

12 → 17 → 20 → [2]

23 → 27 → 30 → [3]

23 → 24 → [4]

23 → 25 → 28 → [5]

12 → 13 → 17 → 21 → 22 → [6]

12 → 13 → 15 → [7]

12 → [8]

13 → 15 → 19 → [9]

13 → 14 → 18 → [10]

23 → 26 → 29 → [11]

Scheme 2   Specific sequences of synthetic intermediates in the preparation of inhibitors 2–11

13.8 mmol). The solution was stirred under Ar for 3 h at room temperature. The solvent was removed *in vacuo* to yield the dihydrochloride salt of compound **22** (148 mg, 97%) as a light-yellow solid (m.p. 242–243 °C).

Synthesis of compound 6

To an ice-cold suspension of compound **22** (23 mg, 0.072 mmol) in distilled dichloromethane (2.3 ml) was added dropwise 1 M BCl_3 in dichloromethane (740 μl, 0.74 mmol). The suspension was stirred at 0 °C for 15 min. The reaction was quenched with water (2 ml) and the mixture was sonicated at room temperature for 30 min. The aqueous layer was removed and freeze-dried. The residue was purified by gel-filtration chromatography (Sephadex LH-20, methanol) to yield compound **6** (25 mg, 93%) as a dihydrochloride salt.

Enzyme kinetics

Trypanothione reductase from *C. fasciculata* (cfTR) was purified from an *Escherichia coli* overexpression system and assayed according to an established protocol [34]. The assays were performed at 22 °C and the assay mixture contained 0.9 nM cfTR, 0.1 M Hepes, pH 7.8, 0.05 mM EDTA, 0.25 mM NADPH and either 12, 24, 50, 100 or 200 μM trypanothione. Inhibitor analyses were conducted at three inhibitor concentrations that were approx. 0.5, 1.0 and 2.0 times the estimated K_i from initial experiments. The steady-state kinetic data were graphically analysed by Lineweaver–Burk analysis and subsequently fitted to the appropriate rate equations using the FORTRAN programs of Cleland [35]. The K_i for competitive inhibitors were estimated by fitting the data to the equation:

$$v = \{V_{max.} \times [S]\}/\{K_m(1 + [I]/K_i) + [S]\}$$

The K_i and K_{ii} for mixed inhibitors were estimated by fitting the data to the equation:

$$v = \{V_{max.} \times [S]\}/\{K_m(1 + [I]/K_i) + [S](1 + [I]/K_{ii})\}$$

When the amount of inhibitor was too limited to carry out a full kinetic analysis, the inhibition constant was assumed to be competitive and estimated with the equation:

$$K_i = \{V_i K_m[I]\}/\{(V_N - V_i)([S] + K_m)\}$$

where V_i is the activity with inhibitor and V_N is the activity without inhibitor.

RESULTS AND DISCUSSION

Like trypanothione, kukoamine A is a terminally difunctionalized polyamine. While both substances are protonated at physiological pH, compound **1** has an additional amine group and is capable of supporting a second positive
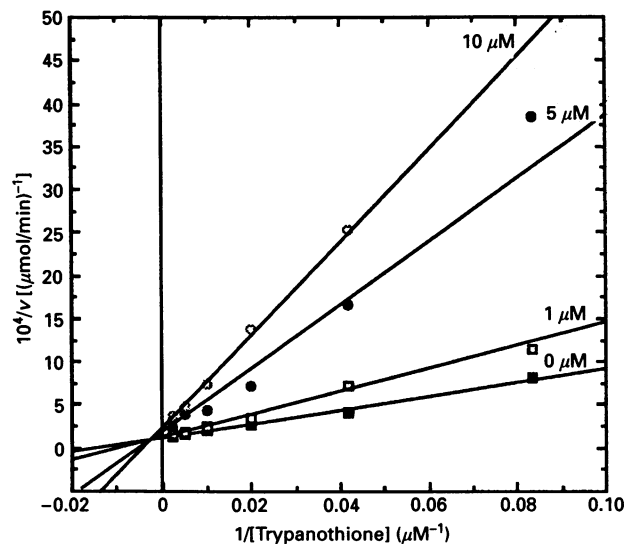
Figure 2   Lineweaver–Burk plot of 1/v versus 1/[S] for kukoamine

The inhibitor concentration used is shown above each line. This plot is representative of other cases of mixed inhibition, except for compound **9**, which produces some non-linearity in the 1/v-versus-1/[S] plot.

Figure 3   Lineweaver–Burk plot of 1/v versus 1/[S] showing competitive inhibition observed with acylspermidine (2)

The inhibitor concentration used is shown above each line. The plot is representative of other cases of competitive inhibition. The observed kinetic behaviour of compond **2** fits equally well with either the competitive and mixed-inhibition mechanism. However, K_{ii} is poorly determined ($K_i = 9.9\ \mu M$; $K_{ii} = 68\ \mu M$); therefore, the competitive mechanism was chosen.

charge. Although the spermine backbone of compound **1** embodies a somewhat longer polyamine chain than the spermidine framework of trypanothione, molecular-modelling studies suggested that the relative distance between the dihydro-caffeoyl amide groups might readily be adjusted by appropriate coiling of the linear polyamine. Assays of kukoamine A against TR revealed a K_i of the order of 1 μM (Figure 2). Against human GR, compound **1** showed no significant inhibition ($K_i > 10$ mM).

Inhibition of TR was not purely competitive. Since catechol ligands are known to shuttle between orthohydroquinone and orthoquinone oxidation states with relative ease, it may be that the observed non-competitive inhibition of TR by compound **1** is due, at least in part, to a redox cycling process. Noteworthy, however, is the fact that several other catechol-containing analogues of compound **1** did exhibit purely competitive inhibition.

Several mono and diacylated polyamine derivatives related to compound **1** were synthesized and screened as inhibitors of TR. Target structures **2–11** (Figure 1) were chosen to explore a range of factors that might affect binding specificity. Factors of interest included the effect of polyamine chain length, the number of acyl substituents, the nature and position of the acyl substituents, and the importance of conformational mobility.

With the exception of derivatives **3** and **9**, all analogues tested in the present study were competitive inhibitors of TR (Figure 3). The chain-shortened spermidine analogue **2** of kukoamine A, with a K_i of 7.5 μM, was nearly as potent an inhibitor of TR as **1** itself (Figure 1). The comparable activity of compounds **1** and **2** might be interpreted in two ways. It may indicate that either polyamine backbone is sufficiently long and flexible to permit both catechol rings to interact with binding pockets on the enzyme. Alternatively, if one dihydrocaffeoyl end group, like one of the tripeptide groups in trypanothione, acts as the principal determinant for ground-state binding in the hydrophobic region of the active site, then the second amide group would be expected to play little part in binding to the enzyme.

To test the latter hypothesis, diacylpolyamines **1** and **2** were compared with the corresponding monoacyl derivatives **5** and **6**.
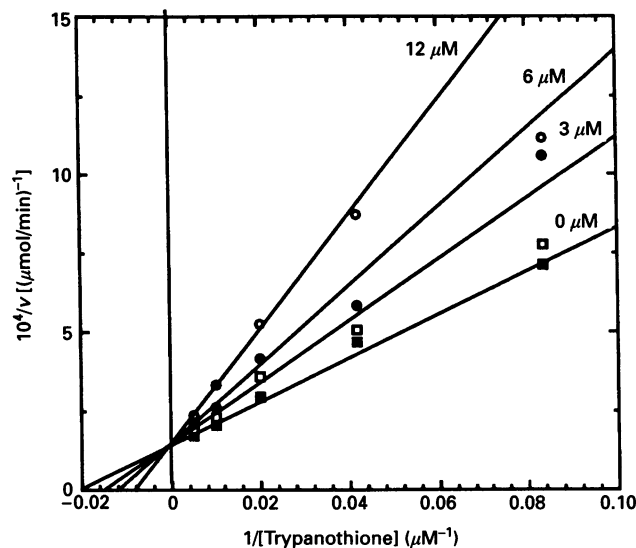
N^1-Dihydrocaffeoylspermine (**5**) was a 50-fold weaker TR inhibitor than compound **1**. The binding of N^1-dihydro-caffeoylspermidine (**6**) also decreased by more than an order of magnitude in comparison with compound **2**. Thus it would appear that both termini of the diacylated spermine and spermidine inhibitors interact with the binding pocket on TR.

This finding also gained support from binding studies designed to test the importance of aryl substituents on the amide end groups. The two catechol rings contribute significantly to binding: replacing them with simple p-methoxyphenyl groups (e.g.compound **1** versus compound **4**; compound **6** versus compound **10**) led to consistently weaker inhibition. Likewise N^1-(p-methoxycinnamoyl)spermidine (**9**), as well as its dihydro derivative (**10**) were much weaker TR inhibitors than catechol (**6**).

Two approaches were used to probe the importance of conformational freedom of the acylpolyamines. In the first approach, a *trans* double bond was introduced in the arylpropanamide group (compare compound **1** with compound **3** and compound **10** with compound **9**) to reduce the rotational mobility at the chain termini. The additional double bond in amides **3** and **9** generates a fully conjugated β-aryl unsaturated amide which significantly decreases the rotational mobility of the polyamine end group. However, this change resulted in no clear-cut trend in inhibitor potency. In the catechol series, bis-unsaturated amide **3** was a slightly weaker inhibitor than kukoamine A and also showed non-competitive inhibition. In the p-methoxyphenyl series, bis-unsaturated amide **9** was a somewhat more potent inhibitor than compound **10**.

In the second approach, a cyclization was carried out using formaldehyde to transform intrachain 1,3-diamine substructures into hexahydropyrimidine rings [28], (compare compound **9** with compound **7**; compound **4** with compound **8**), resulting in a reduction of the overall length and freedom of the polyamine chain. Despite their restricted reach and significantly diminished

rotational freedom, both compounds **7** and **8** still inhibit TR, although the added binding contribution of the second aromatic ring which was noted earlier (compound **1** versus compound **5**; compound **2** versus compound **6**) is no longer evident (cf. compound **7** versus compound **8**).

The γ-glutaminyl residue present in conserved γ-glutaminyl-cysteinyl substructures both in GSSG and in $T(S)_2$ plays a very important role in substrate binding [16,36]. Indeed, if the catechol groups of compound **1** reach all the way to the γ-glutamine-binding pockets, the similarity of carboxy and catechol groups as pharmacophores might explain the enhanced potency of dihydrocaffeoyl-containing inhibitors. We were intrigued by the possibility that a polyamine functionalized at its termini with γ-glutaminyl residues might constitute a potent and selective TR inhibitor, since it would experience the same hydrogen-bonding interactions as the γ-glutamic acid residue in substrate, while being predisposed towards favourable electrostatic interactions with TR. We therefore developed an improved synthesis of N^1N^{12}-bis-(γ-glutaminyl)spermine (**11**), which had earlier been prepared in connection with studies on transglutaminases [37]. Compound **11** displayed a 340-fold weaker affinity for TR than compound **1**, indicating that the γ-glutaminyl groups cannot effectively substitute for the catechols. We interpret this to indicate that the catechol groups do not bind in the γ-glutaminyl-binding pockets, but bind in the more central hydrophobic portion of the $T(S)_2$-binding site.

In summary, a new family of trypanothione reductase inhibitors has been identified from lead compound **1**, which is a naturally occurring polyamine, kukoamine A. Structure–activity relationships in compounds **1–11** reflect the interdependence of the many structural features and the non-additivity of effects. The complexities inherent in uncovering such relationships also emphasize the importance of structural data. Both solid-phase (X-ray) and solution-phase (NMR) structural data can elucidate protein–ligand binding modes and thus aid the interpretation of effects on binding. So far, none of the active compounds has been assayed against intact parasites. Nevertheless, we believe the identification of potent inhibitors, such as compound **1**, of the pure enzyme may provide success in co-crystallization studies with TR, and that structures of enzyme–inhibitor complexes will contribute to the design of effective new antiparasitic agents.

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